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For: Amylase Variants

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Attached please find certified copies of foreign applications from which priority is claimed for this case:

Country: Denmark
Application Number: PA 1995 00126
Filing Date: February 3, 1995

Country: Denmark
Application Number: PA 01097
Filing Date: September 29, 1995

Country: Denmark
Application Number: PA 1995 01121
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Date: October 6, 2004

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Kongeriget Danmark

Patent application No.: PA 1995 00336

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Titlel: Amylase variants

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Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

27 September 2004

Pia Høybye-Olsen

AMYLASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to α-amylase variants having improved properties relative to the parent enzyme (e.g. improved thermal and/or oxidation stability and/or reduced calcium ion dependency), and thereby improved washing and/or dishwashing (and/or textile desizing) performance. The invention also relates to DNA constructs encoding the variants, and to vectors and cells harbouring the DNA constructs. The invention further relates to methods of producing the amylase variants, and to detergent additives and detergent compositions comprising the amylase variants. Furthermore, the invention relates to the use of the amylase variants for textile desizing.

BACKGROUND OF THE INVENTION

α-Amylase enzymes have been used industrially for a number of years and for a variety of different purposes, the most important of which are starch liquefaction, textile desizing, starch modification in the paper and pulp industry, and for brewing and baking. A further use of α-amylases which is becoming increasingly important is the removal of starchy stains during washing or dishwashing.

In recent years attempts have been made to construct α-amylase variants having improved properties with respect to specific uses such as starch liquefaction and textile desizing.

For instance, US 5,093,257 discloses chimeric α-amylases comprising an N-terminal part of a *B. stearothermophilus* α-amylase and a C-terminal part of a *B. licheniformis* α-amylase. The chimeric α-amylases are stated to have unique properties, such as a different thermostability, as compared to their parent α-amylase. However, all of the specifically described chimeric α-amylases were

shown to have a decreased enzymatic activity as compared to their parent α-amylases.

EP 252 666 describes hybrid amylases of the general formula Q-R-L, in which Q is a N-terminal polypeptide residue of from 55 to 60 amino acid residues which is at least 75% homologous to the 57 N-terminal amino acid residues of a specified α-amylase from *B. amyloliquefaciens*, R is a specified polypeptide, and L is a C-terminal polypeptide comprising from 390 to 400 amino acid residues which is at least 75% homologous to the 395 C-terminal amino acid residues of a specified *B. licheniformis* α-amylase.

Suzuki et al. (1989) disclose chimeric α-amylases, in which specified regions of a *B. amyloliquefaciens* α-amylase have been substituted for the corresponding regions of a *B. licheniformis* α-amylase. The chimeric α-amylases were constructed with the purpose of identifying regions responsible for thermostability. Such regions were found to include amino acid residues 177-186 and amino acid residues 255-270 of the *B. amyloliquefaciens* α-amylase. The alterations of amino acid residues in the chimeric α-amylases did not seem to affect properties of the enzymes other than their thermostability.

WO 91/00353 discloses α-amylase mutants which differ from their parent α-amylase in at least one amino acid residue. The α-amylase mutants disclosed in said patent application are stated to exhibit improved properties for application in the degradation of starch and/or textile desizing due to their amino acid substitutions. Some of the mutants exhibit improved stability, but no improvements in enzymatic activity were reported or indicated. The only mutants exemplified are prepared from a parent *B. licheniformis* α-amylase and carry one of the following mutations: H133Y or H133Y + T149I. Another suggested mutation is A111T.

FR 2,676,456 discloses mutants of the *B. licheniformis* α-amylase, in which an amino acid residue in the proximity of His 133 and/or an amino acid residue in the

proximity of Ala 209 have been replaced by a more hydrophobic amino acid residue. The resulting α-amylase mutants are stated to have an improved thermostability and to be useful in the textile, paper, brewing and starch liquefaction industry.

EP 285 123 discloses a method of performing random mutagenesis of a nucleotide sequence. As an example of such sequence a nucleotide sequence encoding a *B. stearothermophilus* α-amylase is mentioned. When mutated, an α-amylase variant having improved activity at low pH values is obtained.

In none of the above references is it mentioned or even suggested that α-amylase mutants may be constructed which have improved properties with respect to the detergent industry.

EP 525 610 relates to mutant enzymes having improved stability towards ionic tensides. The mutant enzymes have been produced by replacing an amino acid residue in the surface part of the parent enzyme with another amino acid residue. The only mutant enzyme specifically described in EP 525 610 is a protease. Amylase is mentioned as an example of an enzyme which may obtain an improved stability towards ionic tensides, but the type of amylase, its origin or specific mutations are not specified.

WO 94/02597 discloses α-amylase mutants which exhibit improved stability and activity in the presence of oxidizing agents. In the mutant α-amylases, one or more methionine residues have been replaced with amino acid residues different from Cys and Met. The α-amylase mutants are stated to be useful as detergent and/or dishwashing additives as well as for textile desizing.

WO 94/18314 discloses oxidatively stable α-amylase mutants, including mutations in the M197 position of *B. licheniformis* α-amylase.

EP 368 341 describes the use of pullulanase and other amylolytic enzymes optionally in combination with an α-amylase for washing and dishwashing.

An object of the present invention is to provide α-amylase variants which - relative to their parent α-amylase - possess improved properties of importance, inter alia, in relation to the washing and/or dishwashing performance of the variants in question, e.g. increased thermal stability, increased stability towards oxidation, reduced dependency on Ca^{2+} ion and/or improved stability or activity in the pH region of relevance in, e.g., laundry washing or dishwashing. Such variant α-amylases have the advantage, among others, that they may be employed in a lower dosage than their parent α-amylase. Furthermore, the α-amylase variants may be able to remove starchy stains which cannot, or can only with difficulty, be removed by α-amylase detergent enzymes known today.

BRIEF DISCLOSURE OF THE INVENTION

A goal of the work underlying the present invention was to improve, if possible, the stability of, *inter alia*, particular α-amylases which are obtainable from *Bacillus* strains and which themselves had been selected on the basis of their starch removal performance in alkaline media (such as in detergent solutions as typically employed in laundry washing or dishwashing) relative to many of the currently commercially available α-amylases. In this connection, the present inventors have surprisingly found that it is in fact possible to improve properties of the types mentioned earlier (*vide supra*) of such a parent α-amylase by judicial modification of one or more amino acid residues in various regions of the amino acid sequence of the parent α-amylase. The present invention is based on this finding.

Accordingly, in a first aspect the present invention relates to variants of a parent α-amylase, the parent α-amylase in question being one which:

i) has one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively, herein; or

ii) displays at least 80% homology with one or more of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively; and/or displays immunological cross-reactivity with an antibody raised against an α-amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively; and/or is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding an α-amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively.

DNA sequences encoding the α-amylase amino acid sequences in question are shown in SEQ ID No. 4 (encoding the amino acid sequence shown in SEQ ID No. 1, SEQ ID No. 5 (encoding the amino acid sequence shown in SEQ ID No. 2) and SEQ ID No. 6 (encoding the amino acid sequence shown in SEQ ID No. 3).

The variants of the invention are variants in which: (a) at least one amino acid residue of the parent α-amylase has been deleted; and/or (b) at least one amino acid residue of the parent α-amylase has been replaced (i.e. substituted) by a different amino acid residue; and/or (c) at least one amino acid residue has been inserted relative to the parent α-amylase. The variants in question have themselves α-amylase activity and exhibit at least one of the following properties relative to the parent α-amylase:

increased thermostability, i.e. satisfactory retention of enzymatic activity at a temperature higher than that suitable for use with the parent enzyme;

increased oxidation stability, i.e. increased resistance to degradation by oxidants (such as oxygen, oxidizing bleaching agents and the like);

reduced Ca^{2+} dependency, i.e. the ability to function satisfactorily in the presence of a lower Ca^{2+} concentration than in the case of the parent α-amylase. α-Amylases with such reduced Ca^{2+} dependency are highly desirable for use in detergent compositions, since such compositions typically contain relatively large amounts of substances (such as phosphates, EDTA and the like) which bind calcium ions strongly.

Examples of other desirable improvements or modifications of properties (relative to the parent α-amylase in question) which may be achieved with a variant according to the invention are:

increased stability and/or α-amylolytic activity at neutral to relatively high pH values, e.g. at pH values in the range of 7-10.5, such as in the range of 8.5-10.5;

increased α-amylolytic activity at relatively high temperatures, e.g. temperatures in the range of 40-70°C;

increase or decrease of the isoelectric point (pI) so as to better match the pI value for the α-amylase variant in question to the pH of the medium (e.g. a laundry washing medium, dishwashing medium or textile-desizing medium) in which the variant is to be employed (*vide infra*).

In the context of the present invention, "improved performance" as used in connection with washing and dishwashing is, as already indicated above, intended to mean improved removal of starchy stains, i.e. stains containing starch, during washing or dishwashing, respectively. The performance may be determined in conventional washing and dishwashing experiments and the improvement evaluated as a comparison with the performance of the parent α-amylase in question. An example of a small-scale "mini dishwashing test" which can be used an indicator of dishwashing performance is described in the Experimental section, below.

It will be understood that a variety of different characteristics of the α-amylase variant, including specific activity, substrate specificity, Km, Vmax, pI, pH optimum, temperature optimum, thermoactivation, stability towards oxidants and detergents, etc., taken alone or in combination are involved in providing the improved performance. The skilled person will be aware that the performance of the variant cannot, alone, be predicted on the basis of the above characteristics, but would have to be accompanied by washing and/or dishwashing performance tests.

In further aspects the invention relates to a DNA construct comprising a DNA sequence encoding an α-amylase variant of the invention, a recombinant expression vector carrying the DNA construct, a cell which is transformed with the DNA construct or the vector, as well as a method of producing an α-amylase variant by culturing such a cell under conditions conducive to the production of the α-amylase variant, after which the α-amylase variant is recovered from the culture.

In a further aspect the invention relates to a method of preparing a variant of a parent α-amylase which by virtue of its improved properties as described above exhibits improved washing and/or dishwashing performance as compared to the parent α-amylase. This method comprises

a) constructing a population of cells containing genes encoding variants of said parent α-amylase,

b) screening the population of cells for α-amylase activity under conditions simulating at least one washing and/or dishwashing condition,

c) isolating a cell from the population containing a gene encoding a variant of said parent α-amylase which has improved activity as compared with the parent α-amylase under the conditions selected in step b),

d) culturing the cell isolated in step c) under suitable conditions in an appropriate culture medium, and

e) recovering the α-amylase variant from the culture obtained in step d).

The invention also relates to a variant (which is a variant according the invention) prepared by the latter method.

In the present context, the term "simulating at least one washing and/or dishwashing condition" is intended to indicate a simulation of, e.g., the temperature or pH prevailing during washing or dishwashing, as well as the chemical composition of a detergent composition to be used in the washing or dishwashing treatment. The term "chemical composition" is intended to include one, or a combination of two or more, constituents of the detergent composition in question. The constituents of a number of different detergent compositions are listed further below.

The "population of cells" referred to in step a) may suitably be constructed by cloning a DNA sequence encoding a parent α-amylase and subjecting the DNA to site-directed or random mutagenesis as described herein.

In the present context the term "variant" is used interchangeably with the term "mutant". The term "variant" is intended to include hybrid α-amylases, i.e. α-amylases comprising parts of at least two different α-amylolytic enzymes. Thus, such a hybrid may be constructed, e.g., from: one or more parts each deriving from a variant as already defined above; or one or more parts each deriving from a variant as already defined above, and one or more parts each deriving from an unmodified parent α-amylase. In this connection, the invention also relates to a method of producing such a hybrid α-amylase having improved washing and/or dishwashing performance as compared to any of its constituent enzymes (i.e. as compared to any of the enzymes which contribute a part to the hybrid), which method comprises:

a) recombining *in vivo* or *in vitro* the N-terminal coding region of an α-amylase gene or corresponding cDNA of one of the constituent α-amylases with the C-terminal coding region of an α-amylase gene or corresponding cDNA of another constituent α-amylase to form recombinants,

b) selecting recombinants that produce a hybrid α-amylase having improved washing and/or dishwashing performance as compared to any of its constituent α-amylases,

c) culturing recombinants selected in step b) under suitable conditions in an appropriate culture medium, and

d) recovering the hybrid α-amylase from the culture obtained in step c).

In further aspects the invention relates to the use of an α-amylase variant of the invention [including any variant or hybrid prepared by one of the above mentioned methods] as a detergent enzyme, in particular for washing or dishwashing, to a detergent additive and a detergent composition comprising the α-amylase variant, and to the use of an α-amylase variant of the invention for textile desizing.

Random mutagenesis may be used to generate variants according to the invention, and the invention further relates to a method of preparing a variant of a parent α-amylase, which method comprises

(a) subjecting a DNA sequence encoding the parent α-amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated amylolytic enzyme which has improved properties as described above (e.g. properties such as decreased calcium dependency, increased oxidation stability, increased thermostability, and/or improved activity at relatively high pH) as compared to the parent α-amylase.

DETAILED DISCLOSURE OF THE INVENTION

Nomenclature

In the present description and claims, the conventional one-letter codes for nucleotides and the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, α-amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, and by way of example, the substitution of alanine for asparagine in position 30 is shown as:

Ala 30 Asn or A30N

a deletion of alanine in the same position is shown as:

Ala 30 * or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala 30 AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, exemplified by amino acid residues 30-33, is indicated as (30-33)*.

Where a specific α-amylase contains a "deletion" (i.e. lacks an amino acid residue) in comparison with other α-amylases and an insertion is made in such a position, this is indicated as:

* 36 Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala 30 Asp + Glu 34 Ser or A30N+E34S

representing mutations in positions 30 and 34 (in which alanine and glutamic acid replace, i.e. are substituted for, asparagine and serine, respectively).

When one or more alternative amino acid residues may be inserted in a given position this is indicated as:
A30N,E or
A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in that position. Thus, for instance, when a modification (replacement) of a methionine in position 202 is mentioned, but not specified, it is to be understood that any of the other amino acids may be substituted for the methionine, i.e. any other amino acid chosen among A,R,N,D,C,Q,E,G,H,I,L,K,F,P,S,T,W,Y and V.

The parent α-amylase

As already indicated, an α-amylase variant of the invention is very suitably prepared on the basis of a parent α-amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively (*vide infra*).

The parent α-amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, are obtainable from alkaliphilic *Bacillus* strains (strain NCIB 12512 and strain NCIB 12513, respectively), both of which are described in detail in EP 0 277 216 B1 and were deposited in accordance with the Budapest Treaty on 5 August 1987 at the National Collection of Industrial Bacteria (NCIB) under accession Nos. NCIB 12512 and NCB 12513, respectively. Their preparation from those sources is described in greater detail in the Experimental section herein (*vide infra*).

The parent α-amylase having the amino acid sequence shown in SEQ ID No. 3 is obtainable from *Bacillus stearothermophilus* and is described in, inter alia, J. Bacteriol. 166 (1986) pp. 635-643.

Apart from variants of the above-mentioned parent α-amylases having the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively, other interesting variants according to the invention include variants of parent α-amylases which have amino acid sequences exhibiting a high degree of homology, such as at least 70% homology, preferably (as already indicated) at least 80% homology, desirably at least 85% homology, and more preferably at least 90% homology, e.g. ≥95% homology, with at least one of the latter three amino acid sequences.

As also already indicated above, further criteria for identifying a suitable parent α-amylase are a) that the α-amylase displays an immunological cross-reaction with an antibody raised against an α-amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively, and/or b) that the α-amylase is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding an α-amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively.

With regard to determination of the degree of homology of polypeptides (such as enzymes), amino acid sequence comparisons can be performed using known algorithms, such as the one described by Lipman and Pearson (1985).

Assays for immunological cross-reactivity may be carried out using an antibody raised against, or reactive with, at least one epitope of the α-amylase having the amino acid sequence shown in SEQ ID No. 1, or of the α-amylase having the amino acid sequence shown in SEQ ID No. 2, or of the α-amylase having the amino acid sequence shown in SEQ ID No. 3.

The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al. (1989). Examples of suitable assay techniques well known in the art include Western

Blotting and Radial Immunodiffusion Assay, e.g. as described by Hudson et al. (1989).

The oligonucleotide probe for use in the identification of suitable parent α-amylases on the basis of probe hybridization [criterion b) above] may, by way of example, suitably be prepared on the basis of the full or partial amino acid sequence of an α-amylase having one of the sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively, or on the basis of the full or partial nucleotide sequence corresponding thereto (SEQ ID No. 4, SEQ ID No. 5 or SEQ ID No. 6, respectively).

Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50μg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100μM ATP for 18h at ~40°C, or using other methods described by, e.g., Sambrook et al. (1989).

Influence of mutations on particular properties

From the results obtained by the present inventors it appears that changes in a particular property, e.g. thermal stability or oxidation stability, exhibited by a variant relative to the parent α-amylase in question can to a considerable extent be correlated with the type of, and positioning of, mutation(s) (amino acid substitutions, deletions or insertions) in the variant. It is to understood, however, that the observation that a particular mutation or pattern of mutations leads to changes in a given property in no way excludes the possibility that the mutation(s) in question can also influence other properties.

Oxidation stability: With respect to increasing the oxidation stability of an α-amylase variant relative to its parent α-amylase, it appears to be particularly desirable that at least one, and preferably multiple, oxidizable amino acid residue(s) of the parent has/have been deleted or replaced (i.e. substituted by) a

different amino acid residue which is less susceptible to oxidation than the original oxidizable amino acid residue.

Particularly relevant oxidizable amino acid residues in this connection are cysteine, methionine, tryptophan and tyrosine. Thus, for example, in the case of parent α-amylases containing cysteine it is anticipated that deletion of cysteine residues, or substitution thereof by less oxidizable amino acid residues, will be of importance in obtaining variants with improved oxidation stability relative to the parent α-amylase.

In the case of the above-mentioned parent α-amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, both of which contain no cysteine residues but have a significant methionine content, the deletion or substitution of methionine residues is particularly relevant with respect to achieving improved oxidation stability of the resulting variants. Thus, deletion or substitution [e.g. by threonine (T), or by one of the other amino acids listed above] of one or more of the methionine residues in positions M9, M10, M105, M202, M208, M261, M309, M382, M430 and M440 of the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, and/or in position M323 of the amino acid sequence shown in SEQ ID No. 2 (or deletion or substitution of methionine residues in equivalent positions in the sequence of another α-amylase meeting one of the other criteria for a parent α-amylase mentioned above) appear to be particularly effective with respect to increasing the oxidation stability.

In the case of the parent α-amylase having the amino acid sequence shown in SEQ ID No. 3, relevant amino acid residues which may be deleted or substituted with a view to improving the oxidation stability include the single cysteine residue (C363) and - by analogy with the sequences shown in SEQ ID No. 1 and SEQ ID No. 3 - the methionine residues located in positions M8, M9, M96, M200, M206, M284, M307, M311, M316 and M438.

In this connection, the term "equivalent positions" denotes positions which, on the basis of an alignment of the amino acid sequence of the parent α-amylase in question with the "reference" α-amylase amino acid sequence in question (for example the sequence shown in SEQ ID No. 1) so as to achieve juxtapositioning of amino acid residues/regions which are common to both, correspond to the above-mentioned positions in the reference sequence in question.

Particularly interesting mutations in connection with modification of the oxidation stability of the α-amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, are one or more of the following methionine substitutions (or equivalents thereof in the amino acid sequences of other α-amylases meeting the requirements of a parent α-amylase in the context of the invention): M202A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V. Further relevant methionine substitutions in the amino acid sequence shown in SEQ ID No. 2 are: M323A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V.

Particularly interesting mutations in connection with modification of the oxidation stability of the α-amylase having the amino acid sequence shown in SEQ ID No. 3 are one or more of the following methionine substitutions:
M200A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V;
M311A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V; and
M316A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V.

Thermal stability: With respect to increasing the thermal stability of an α-amylase variant relative to its parent α-amylase, it appears to be particularly desirable to delete at least one, and preferably two or even three, of the following amino acid residues in the amino acid sequence shown in SEQ ID No. 1 (or their equivalents): F180, R181, G182, T183, G184 and K185. The corresponding, particularly relevant (and equivalent) amino acid residues in the amino acid sequences shown in SEQ ID No. 2 and SEQ ID No. 3, respectively, are: F180, R181, G182, D183, G184 and K185 (SEQ ID No. 2); and F178, R179, G180, I181, G182 and K183 (SEQ ID No. 3).

Particularly interesting pairwise deletions of this type are as follows:
R181* + G182*; and T183* + G184* (SEQ ID No. 1);
R181* + G182*; and D183* + G184* (SEQ ID No. 2); and
R179* + G180*; and I181* + G182* (SEQ ID No. 3)
(or equivalents of these pairwise deletions in another α-amylase meeting the requirements of a parent α-amylase in the context of the present invention).

Other mutations which appear to be of importance in connection with thermal stability are one or more of the following substitutions in the amino acid sequence shown in SEQ ID No. 1 (or their equivalents): P260E; R124P; M105F,I,L,V; M208F,W,Y; L217I; V206I,L,F.

For the parent α-amylase having the amino acid sequence shown in SEQ ID No. 2, important further mutations are, correspondingly, one or more of the substitutions: M105F,I,L,V; M208F,W,Y; L217I; and V206I,L,F.

For the parent α-amylase having the amino acid sequence shown in SEQ ID No. 3, important further mutations are one or both of the substitutions: M206F,W,Y; and L215I.

Ca^{2+} dependency: With respect to decreasing the Ca^{2+} dependency of an α-amylase variant relative to its parent α-amylase, it appears to be particularly desirable to incorporate one or more of the following substitutions in the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2 (or an equivalent substitution in another parent α-amylase within the context of the invention): Y243F, K108R, K179R, K239R, K242R, K269R, D163N, D188N, D192N, D199N, D205N, D207N, D209N, E190Q, E194Q and N106D.

In the case of the amino acid sequence shown in SEQ ID No. 3, particularly desirable substitutions appear to be one or more of the following; K107R, K177R, K237R, K240R, D162N, D186N, D190N, D197N, D203N, D205N, D207N, E188Q and E192Q.

As well as the above-mentioned replacements of D residues with N residues, or of E residues with Q residues, other relevant substitutions in the context of reducing Ca^{2+} dependency are replacement of the D and/or E residues in question with any other amino acid residue.

Isoelectric point (pI): Preliminary results indicate that the washing performance, e.g. the laundry washing performance, of an α-amylase is optimal when the pH of the washing liquor (washing medium) is close to the pI value for the α-amylase in question. It will thus be desirable, where appropriate, to produce an α-amylase variant having an isoelectric point (pI value) which is better matched to the pH of a medium (such as a washing medium) in which the enzyme is to be employed than the isoelectric point of the parent α-amylase in question.

With respect to decreasing the isoelectric point, preferred mutations in the amino acid sequence shown in SEQ ID No. 1 include one or more of the following substitutions: Q86E, R124P, S154D, T183D, V222E, P260E, R310A, Q346E, N437E, K444Q and R452H. Correspondingly, preferred mutations in the amino acid sequence shown in SEQ ID No. 3 include one or more of the substitutions: L85E, S153D, I181D, K220E, P258E, R308A, P344E, Q358E and S435E.

With respect to increasing the isoelectric point, preferred mutations in the amino acid sequence shown in SEQ ID No. 2 include one or more of the following substitutions: E86Q,L; D154S; D183T,I; E222V,K; E260P; A310R; E346Q,P; E437N,S; and H452R.

In the Experimental section below, the construction of a number of variants according to the invention is described.

α-Amylase variants of the invention will, apart from having one or more improved properties as discussed above, preferably be such that they have a higher starch hydrolysis velocity at low substrate concentrations than the parent α-amylase. Alternatively, an α-amylase variant of the invention will preferably be one which

has a higher Vmax and/or a lower Km than the parent α-amylase when tested under the same conditions. In the case of a hybrid α-amylase, the "parent α-amylase" to be used for the comparison should be the one of the constituent enzymes having the best performance.

The Vmax, Km and V may be determined by well-known procedures.

Methods of preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene. For region-specific random mutagenesis with a view to improving the thermal stability, the following codon positions, in particular, may appropriately be targetted (using one-letter amino acid abbreviations):

In the amino acid sequence shown in SEQ ID No. 1:

120-140 = VEVNRSNRNQETSGEYAIEAW

178-187 = YKFRGTGKAW

264-275 = VAEFWKNDLGAIEN

In the amino acid sequence shown in SEQ ID No. 2:

120-140 = VEVNPNNRNQEISGDYTIEAW

178-187 = YKFRGDKAW

264-275 = VAEFWKNDLGALEN

In the amino acid sequence shown in SEQ ID No. 3:

119-139 = VEVNPSDRNQEISGTYQIQAW

176-185 = YKFRGIGKAW

262-273 = VGEYWSYDINKLHN

With a view to achieving reduced Ca^{2+} dependency, the following codon positions, in particular, may appropriately be targetted:

In the amino acid sequence shown in SEQ ID No. 1:
178-209 = YKFRGTGKAWDWEVDTENGNYDYLMYADVDMD
237-246 = AVKHIKYSFT

In the amino acid sequence shown in SEQ ID No. 2:
178-209 = YKFRGDGKAWDWEVDSENGNYDYLMYADVDMD
237-246 = AVKHIKYSFT

The random mutagenesis of a DNA sequence encoding a parent α-amylase to be performed in accordance with step a) of the above-described method of the invention may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the

mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the amylolytic enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α-amylase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the amylolytic enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent amylolytic enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA

sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step (b) or the screening step (c) being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gramnegative bacteria such as *E.coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis: the random mutagenesis may advantageously be localized to a part of the parent α-amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may

normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above or any other suitable technique known in the art.

Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently performed by use of aa filter assay based on the following principle:

A microorganism capable of expressing the mutated amylolytic enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the micro-organisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The topfilter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the

conditions to be used for screening or may be treated during the detection of enzymatic activity.

The enzymatic activity may be detected by a dye, flourescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

α-Amylase activity is detected by Cibacron Red labelled amylopectin, which is immobilized on agarose. For screening for variants with increased thermal and high-pH stability, the filter with bound α-amylase variants is incubated in a buffer at pH 10.5 and 60° or 65°C for a specified time, rinsed briefly in deionized water and placed on the amylopectin-agarose matrix for activity detection. Residual activity is seen as lysis of Cibacron Red by amylopectin degradation. The conditions are chosen to be such that activity due to the α-amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected. Stabilized variants show, under the same conditions, increased colour intensity due to increased liberation of Cibacron Red.

For screening for variants with an activity optimum at a lower temperature and/or over a broader temperature range, the filter with bound variants is placed directly on the amylopectin-Cibacron Red substrate plate and incubated at the desired temperature (e.g. 4°C, 10°C or 30°C) for a specified time. After this time activity due to the α-amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected, whereas variants with optimum activity at a lower temperature will show increase amylopectin lysis. Prior to incubation onto the amylopectin matrix, incubation in all kinds of desired media - e.g. solutions containing Ca^{2+}, detergents, EDTA or other relevant additives - can be carried out

in order to screen for changed dependency or for reaction of the variants in question with such additives.

Methods of preparing hybrid α-amylases

As an alternative to site-specific mutagenesis, α-amylase variants which are hybrids of at least two constituent α-amylases may be prepared by combining the relevant parts of the respective genes in question.

Naturally occurring enzymes may be genetically modified by random or site directed mutagenesis as described above. Alternatively, part of one enzyme may be replaced by a part of another to obtain a chimeric enzyme. This replacement can be achieved either by conventional *in vitro* gene splicing techniques or by *in vivo* recombination or by combinations of both techniques. When using conventional *in vitro* gene splicing techniques, a desired portion of the α-amylase gene coding sequence may be deleted using appropriate site-specific restriction enzymes; the deleted portion of the coding sequence may then be replaced by the insertion of a desired portion of a different α-amylase coding sequence so that a chimeric nucleotide sequence encoding a new α-amylase is produced. Alternatively, α-amylase genes may be fused, e.g. by use of the PCR overlay extension method described by Higuchi et al. 1988.

The *in vivo* recombination techniques depend on the fact that different DNA segments with highly homologous regions (identity of DNA sequence) may recombine, i.e. break and exchange DNA, and establish new bonds in the homologous regions. Accordingly, when the coding sequences for two different but homologous amylase enzymes are used to transform a host cell, recombination of homologous sequences *in vivo* will result in the production of chimeric gene sequences. Translation of these coding sequences by the host cell will result in production of a chimeric amylase gene product. Specific *in vivo* recombination techniques are described in US 5,093,257 and EP 252 666.

Alternatively, the hybrid enzyme may be synthesized by standard chemical methods known in the art. For example, see Hunkapiller et al. (1984). Accordingly, peptides having the appropriate amino acid sequences may be synthesized in whole or in part and joined to form hybrid enzymes (variants) of the invention.

Expression of α-amylase variants

According to the invention, a mutated α-amylase-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are

the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus Amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular.

Procedures suitable for constructing vectors of the invention encoding an α-amylase variant, and containing the promoter, terminator and other elements, respectively, are well known to persons skilled in the art [cf., for instance, Sambrook et al. (1989)].

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae*

or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

Owing to their activity at alkaline pH values, α-amylase variants of the invention are well suited for use in a variety of industrial processes. In particular, they find potential applications as a component in washing, dishwashing and hard surface cleaning detergent compositions (*vide infra*), but may also be useful in the production of sweeteners and ethanol from starch. Conditions for conventional starch-converting processes and liquefaction and/or saccharification processes are described in, for instance, US 3,912,590, EP 252,730 and EP 63,909. α-Amylase variants of the invention also possess properties of value in the

production of lignocellulosic materials, such as pulp, paper and cardboard, from starch-reinforced waste paper and waste cardboard, especially where repulping occurs at a pH above 7, and where amylases can facilitate the disintegration of the waste material through degradation of the reinforcing starch. α-Amylase variants of the invention are well suited for use in the deinking/recycling processes of making paper out of starch-coated or starch-containing waste printed paper. It is usually desirable to remove the printing ink in order to produce new paper of high brightness; examples of how the variants of the invention may be used in this way are described in PCT/DK94/00437.

The α-amylases of the invention may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such as calcium carbonate, kaolin and clays. With the alkaline α-α-amylase variants of the invention it is feasible to modify the starch in the presence of the filler, thus allowing for a simpler, integrated process.

The α-amylase variants of the invention are also well suited for use in textile desizing. In the textile processing industry, α-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in subsequent processes in which the fabric is scoured, bleached and dyed. Enzymatic starch degradation is preferred because it does not harm the fibres of the textile or fabric.

In order to reduce processing costs and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional α-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fibre damage because of the rather aggressive chemicals used.

Accordingly, it would be desirable to use an α-amylase variant of the invention, having improved performance in alkaline solution. The α-amylase variant may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

α-Amylase variants of the invention are also believed to be very useful in beer-making processes; in such processes the variants will typically be added during the mashing process.

Detergent Additive and Composition for Dishwashing and Washing

Due to the improved washing and/or dishwashing performance which will often be a consequence of improvements in properties as discussed above, numerous α-amylase variants (including hybrids) of the invention are particularly well suited for incorporation into detergent compositions, e.g. detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11. According to the invention, the α-amylase variant may be added as a component of a detergent composition. As such, it may be included in the detergent composition in the form of a detergent additive. The detergent composition as well as the detergent additive may additionally comprise one or more other enzymes conventionally used in detergents, such as proteases, lipases, amylolytic enzymes, oxidases (including peroxidases), or cellulases.

It has been found that substantial improvements in washing and/or dishwashing performance may be obtained when α-amylase is combined with another amylolytic enzyme, such as a pullulanase, an iso-amylase, a beta-amylase, an amyloglucosidase or a CTGase. Examples of commercially available amylolytic enzymes suitable for the given purpose are AMG®, Novamyl® and Promozyme®, all available from Novo Nordisk A/S.

Accordingly, in a particular embodiment the invention relates to a detergent additive comprising an α-amylase variant of the invention in combination with at least one other amylolytic enzyme (e.g. chosen amongst those mentioned above).

In a specific aspect, the invention provides a detergent additive. The enzymes may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separated additive or a combined additive, can be formulated, e.g., as a granulate, liquid, slurry, etc. Preferred detergent additive formulations are granulates (in particular non-dusting granulates), liquids (in particular stabilized liquids), slurries or protected enzymes.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation.

Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

In a still further aspect, the invention relates to a detergent composition comprising an α-amylase variant (including hybrid) of the invention.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% of water and 0-20% of organic solvent, or non-aqueous, e.g. as described in EP Patent 120,659.

Detergent Compositions

An α-amylase variant of the invention may typically be a component of a detergent composition, e.g., a laundry detergent composition or a dishwashing detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or amphoteric (zwitterionic). The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), alcohol propoxylate, carboxylated alcohol ethoxylates, nonylphenol

ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as pullulanase, esterase, lipase, cutinase, protease, cellulase, peroxidase, or oxidase, e.g., laccase.

Normally the detergent contains 1-65% of a detergent builder (although some dishwashing detergents may contain even up to 90% of a detergent builder) or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent builders may be subdivided into phosphorus-containing and non-phosphorous-containing types. Examples of phosphorus-containing inorganic alkaline detergent builders include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, polyphosphates and phosphonates. Examples of non-phosphorus-containing inorganic builders include water-soluble alkali metal carbonates, borates and silicates, as well as layered disilicates and the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best known representatives.

Examples of suitable organic builders include alkali metal, ammonium or substituted ammonium salts of succinates, malonates, fatty acid malonates, fatty acid sulphonates, carboxymethoxy succinates, polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates and polyacetyl carboxylates.

The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC; typically in the form of the sodium salt), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, polymaleates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. The bleaching agents may be coated or encapsulated. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite or hypobromite as well as chlorinated trisodium phosphate. The bleaching system may also comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS).

Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable. The bleaching system may also comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

In dishwashing detergents the oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED or NOBS.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative

such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type (as described in EP 0 544 777 B1) or the boronic acid type.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, deflocculant material, foam boosters/foam depressors (in dishwashing detergents foam depressors), suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, dehydrating agents, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of laundry detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaAlSiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%

TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaAlSiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaAlSiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%

Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaAlSiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%

Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaAlSiO_4$)	18 - 32%

Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzene-sulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaAlSiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaAlSiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O,2SiO_2$)	4 - 8%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%

Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

Particular forms of dishwashing detergent compositions within the scope of the invention include:

1) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.4 - 2.5%
Sodium metasilicate	0 - 20%
Sodium disilicate	3 - 20%
Sodium triphosphate	20 - 40%
Sodium carbonate	0 - 20%
Sodium perborate	2 - 9%
Tetraacetylethylenediamine (TAED)	1 - 4%
Sodium sulphate	5 - 33%
Enzymes	0.0001 - 0.1%

2) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 2%
Sodium disilicate	2 - 30%
Sodium carbonate	10 - 50%
Sodium phosphonate	0 - 5%
Trisodium citrate dihydrate	9 - 30%
Nitrilotrisodium acetate (NTA)	0 - 20%
Sodium perborate monohydrate	5 - 10%
Tetraacetylethylenediamine (TAED)	1 - 2%
Polyacrylate polymer (e.g. maleic acid/acrylic acid copolymer)	6 - 25%
Enzymes	0.0001 - 0.1%
Perfume	0.1 - 0.5%
Water	5 - 10

3) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.5 - 2.0%
Sodium disilicate	25 - 40%
Sodium citrate	30 - 55%

Sodium carbonate	0 - 29%
Sodium bicarbonate	0 - 20%
Sodium perborate monohydrate	0 - 15%
Tetraacetylethylenediamine (TAED)	0 - 6%
Maleic acid/acrylic acid copolymer	0 - 5%
Clay	1 - 3%
Poly(amino acids)	0 - 20%
Sodium polyacrylate	0 - 8%
Enzymes	0.0001 - 0.1%

4) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1 - 2%
Zeolite MAP	15 - 42%
Sodium disilicate	30 - 34%
Sodium citrate	0 - 12%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	7 - 15%
Tetraacetylethylenediamine (TAED)	0 - 3%
Polymer	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 5%
Organic phosphonate	0 - 4%
Clay	1 - 2%
Enzymes	0.0001 - 0.1%
Sodium sulphate	Balance

5) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1 - 7%

Sodium disilicate	18 - 30%
Trisodium citrate	10 - 24%
Sodium carbonate	12 - 20%
Monopersulphate (2 $KHSO_5.KHSO_4.K_2SO_4$)	15 - 21%
Bleach stabilizer	0.1 - 2%
Maleic acid/acrylic acid copolymer	0 - 6%
Diethylenetriaminepentaacetate, pentasodium salt	0 - 2.5%
Enzymes	0.0001 - 0.1%
Sodium sulphate, water	Balance

6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

Nonionic surfactant	0 - 1.5%
Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%
C_{13}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 10%
C_{12}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 5%
C_{13}-C_{15} ethoxylated alcohol with an average degree of ethoxylation of 12	0 - 5%
A blend of C_{12}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 9	0 - 6.5%
A blend of C_{13}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 30	0 - 4%
Sodium disilicate	0 - 33%
Sodium tripolyphosphate	0 - 46%

Sodium citrate	0 - 28%
Citric acid	0 - 29%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	0 - 11.5%
Tetraacetylethylenediamine (TAED)	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 7.5%
Sodium sulphate	0 - 12.5%
Enzymes	0.0001 - 0.1%

7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Alkali metal silicate	3.0 - 15.0%
Alkali metal phosphate	20.0 - 40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0 - 45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a C_{16}-C_{18} alkanol)	0.5 - 7.0%
Foam suppressor (e.g. silicone)	0 - 1.5%
Enzymes	0.0001 - 0.1%

8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Sodium silicate	3.0 - 15.0%
Alkali metal carbonate	7.0 - 20.0%
Sodium citrate	0.0 - 1.5%
Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
Low molecule weight polyacrylate polymer	5.0 - 15.0%

Clay gel thickener (e.g. bentonite)	0.0 - 10.0%
Hydroxypropyl cellulose polymer	0.0 - 0.6%
Enzymes	0.0001 - 0.1%
Liquid carrier selected from higher lycols, polyglycols, polyoxides and glycol ethers	Balance

9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

C_{12}-C_{14} fatty acid	0 - 0.5%
Block co-polymer surfactant	1.5 - 15.0%
Sodium citrate	0 - 12%
Sodium tripolyphosphate	0 - 15%
Sodium carbonate	0 - 8%
Aluminium tristearate	0 - 0.1%
Sodium cumene sulphonate	0 - 1.7%
Polyacrylate thickener	1.32 - 2.5%
Sodium polyacrylate	2.4 - 6.0%
Boric acid	0 - 4.0%
Sodium formate	0 - 0.45%
Calcium formate	0 - 0.2%
Sodium n-decydiphenyl oxide disulphonate	0 - 4.0%
Monoethanol amine (MEA)	0 - 1.86%
Sodium hydroxide (50%)	1.9 - 9.3%
1,2-Propanediol	0 - 9.4%
Enzymes	0.0001 - 0.1%
Suds suppressor, dye, perfumes, water	Balance

10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

Alcohol ethoxylate	0 - 20%
Fatty acid ester sulphonate	0 - 30%

Sodium dodecyl sulphate	0 - 20%
Alkyl polyglycoside	0 - 21%
Oleic acid	0 - 10%
Sodium disilicate monohydrate	18 - 33%
Sodium citrate dihydrate	18 - 33%
Sodium stearate	0 - 2.5%
Sodium perborate monohydrate	0 - 13%
Tetraacetylethylenediamine (TAED)	0 - 8%
Maleic acid/acrylic acid copolymer	4 - 8%
Enzymes	0.0001 - 0.1%

11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

Sodium silicate	5 - 10%
Tetrapotassium pyrophosphate	15 - 25%
Sodium triphosphate	0 - 2%
Potassium carbonate	4 - 8%
Protected bleach particles, e.g. chlorine	5 - 10%
Polymeric thickener	0.7 - 1.5%
Potassium hydroxide	0 - 2%
Enzymes	0.0001 - 0.1%
Water	Balance

11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

12) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

An α-amylase variant of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the α-amylase variant may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of α-amylase per liter of wash/dishwash liquor.

Textile desizing

In the textile processing industry, α-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving.

Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch break-down is preferred because it does not involve any harmful effect on the fibre material.

In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional α-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fibre damage because of the rather aggressive chemicals used.

Accordingly, it would be desirable to use α-amylase enzymes having an improved resistance towards or compatible with oxidation (bleaching) agents at elevated pH, in order to retain the advantages of enzymatic size break down in a time-saving simultaneous desizing/scouring/bleaching process.

Thus, α-amylase variants of the invention having improved resistance towards oxidation agents will be useful in desizing processes as described above, in particular for replacement of non-enzymatic alkali or oxidation agents used today.

The present invention is further described with reference to the appended drawing, in which Fig. 1 is a restriction map of plasmid pTVB106.

EXPERIMENTAL SECTION

Preparation of the parent α-amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, from Bacillus strain NCIB 12512 and NCIB 12513, respectively.

Fermentation:

Each of the above-mentioned Bacillus strains was incubated at 26°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of BP-X medium + 0.1 M Carbonate buffer pH 9.0.

BP-X medium:

Potato starch	100 g
Ground barley	50 g
Soybean flour	20 g
Sodium caseinate	10 g
$Na_2HPO_4.12\ H_2O$	9 g
Termamyl® 60L*	0.1 g
Pluronic®	0.1 g

*) available from Novo Nordisk A/S.

The starch in the medium was liquified by slowly heating the medium from 60°C to 85°C for 30 minutes. After this the temperature of the medium was quickly raised to 95°C for 10 minutes, and the medium was then cooled. Lastly, the medium was sterilized by heating at 121°C for 40 minutes.

Purification of the α-amylase from strain NCIB 12512

After 5 days of incubation the culture broth was filtered and concentrated using a Filtron™ ultrafiltration module with 3KD membranes and washed with deionized water until the conductivity was 1 mS/cm. The pH was adjusted to pH 5.9 with 10% (v/v) acetic acid. A S-Sepharose FF column was equilibrated in EKV-buffer, pH 5.9. If not otherwise stated, the purification buffer was 100 mM boric acid,

10 mM succinic acid, 2 mM $CaCl_2$, (EKV-buffer) adjusted to the indicated pH with NaOH.

The enzyme solution was applied to the column, the column was washed with EKV-buffer, pH 5.9, and the amylase was eluted with a linear NaCl gradient (0 -> 500 mM NaCl). Amylase-containing fractions were pooled and the pH adjusted to pH 7 with 3% (w/v) NaOH.

A chelate agarose column was loaded with Cu^{2+} and equilibrated in the following manner: 50 mM $CuSO_4$, pH 5, was pumped on to the column until the whole column was blue, then excess of Cu^{2+} ions were removed by washing the column with 500 mM imidazole, pH 7, and finally the column was equilibrated with EKV-buffer, pH 7. The amylase pool from the S-Sepharose column was applied to the Cu^{2+}-loaded chelate agarose column, the column was washed with EKV-buffer, pH 7, and the enzyme was eluted with a linear gradient of imidazole (0 -> 500 mM imidazole). Amylase-containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a final concentration of 1M $(NH_4)_2SO_4$ in the pool.

A phenyl sepharose column was equilibrated in EKV-buffer + 1M $(NH_4)_2SO_4$, pH 7. The amylase pool from the Cu^{2+} column was applied to the hydrophobic interaction column. Binding experiments had shown that the amylase is a rather hydrophobic enzyme, and hence binds tightly to the phenyl column. Protein which did not bind as tightly to the column was washed off the column with EKV-buffer, pH 7. The amylase was step-eluted from the column with EKV-buffer + 25% (v/v) isopropanol. The amylase-containing pool was adjusted to pH 9.5 with 3% (w/v) NaOH and diluted 5 times with deionized water.

A Q-Sepharose HP column was equilibrated in 20 mM Tris-HCl, pH 9.5. The amylase pool from the phenyl sepharose column was applied to the column and the column was washed with 20 mM Tris-HCl, pH 9.5. The amylase was eluted

with a linear gradient of NaCl (0 -> 250 mM NaCl). The amylase peak was adjusted to pH 7 with 10% (v/v) acetic acid.

A Cu^{2+}-loaded chelating Sepharose FF column (loaded with Cu^{2+} as described for the chelate agarose column) was equilibrated with EKV-buffer, pH 7. The amylase peak from the Q-Sepharose column was applied to the column, and the column was washed thoroughly with EKV-buffer, pH 7. The amylase was eluted with a steep linear gradient of imidazole (0 -> 500 mM imidazole).

The purified amylase was purity checked by SDS-PAGE electrophoresis. The Coomassie-stained gel had only one band.

Purification of the *α*-amylase from strain NCIB 12513

After 5 days of incubation the culture broth was filtered and concentrated using a Filtron™ ultrafiltration module with 3KD membranes. The concentrated solution was filtered and saturated to 20% w/w with ammonium sulfate. The solution was then batch adsorbed using a AFFI-T™ matrix from Kem-En-Tec A/S, Copenhagen, Denmark. The amylase was eluted using 25% isopropanol in 20 mM Tris, pH 7.5, after washing of the matrix with deionized water. The eluted enzyme was subjected to dialysis (20 mM Tris, pH 8.5) and a stepwise batch adsorption on Q-Sepharose FF for colour removal was carried out.

A chelate agarose column was loaded with Cu^{2+} and equilibrated in the following manner: 50 mM $CuSO_4$, pH 5 was pumped on to the column until the whole column was blue, then excess of Cu^{2+} ions was removed by washing the column with 500 mM imidazole, pH 7, and finally the column was equilibrated with 50 mM borate buffer, pH 7.

In spite of the low pI of this amylase (5.8), it was not bound to the Q-Sepharose FF at pH 8.5.

The run-through from the Q-Sepharose FF column was applied on the Cu^{2+}-chelating agarose and eluted using 250 mM imidazole, 20 mM Tris, pH 7.0 and the eluted column was dialysed against 50 mM borate buffer, pH 7.0. The pH was adjusted to pH 9.5, and the dialysed solution was bound on a Q-Sepharose HP and eluted over 10 columns using a linear gradient from 0 -> 250 mM NaCl. Amylase-containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a final concentration of 20% w/w, whereafter the fractions were applied on a phenyl Sepharose column. The column was washed using deionized water and eluted using 25% isopropanol in 50 mM borate buffer, pH 7.0.

The purified amylase was purity checked by SDS-PAGE electrophoresis. The Coomassie-stained gel had only one band.

Physical/chemical properties of the parent α-amylases

As already indicated, the α-amylases from strain NCIB 12512 and NCIB 12513 have the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively. Amino acid sequencing of the two α-amylases was carried out using standard methods [see, e.g., Findley and Geisow (Eds.), Protein Sequencing - A Practical Approach, IRL Press (1989)].

Some other properties of the two α-amylases (prepared and purified as described above) are as follows:

SEQ ID No. 1: pI about 8.8-9.0 (determined by isoelectric focusing on LKB Ampholine™ PAG plates); molecular weight approximately 55 kD (determined by SDS-PAGE).

SEQ ID No. 2: pI about 5.8 (determined by isoelectric focusing on LKB Ampholine™ PAG plates); molecular weight approximately 55 kD (determined by SDS-PAGE).

Determination of α-amylase activity

α-Amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

For the determination of every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The α-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α-amylase activity.

It is important that the measured 620 nm absorbance after 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion.

Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α-amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α-amylase protein) of the α-amylase in question under the given set of conditions. Thus testing different α-amylases of interest (including a reference α-amylase, in this case the parent α-amylase in question) under identical conditions, the specific activity of each of the α-amylases at a given temperature and at a given pH can be compared directly, and the ratio of the specific activity of each

of the α-amylases of interest relative to the specific activity of the reference α-amylase can be determined.

Mini dishwashing assay

The following mini dishwashing assay was used: A suspension of starchy material was boiled and cooled to 20°C. The cooled starch suspension was applied on small, individually identified glass plates (approx. 2 x 2 cm) and dried at a temperature in the range of 60-140°C in a drying cabinet. The individual plates were then weighed. For assay purposes, a solution of standard European-type automatic dishwashing detergent (5 g/l) having a temperature of 55°C was prepared. The detergent was allowed a dissolution time of 1 minute, after which the α-amylase in question was added to the detergent solution (contained in a beaker equipped with magnetic stirring) so as to give an enzyme concentration of 0.5 mg/ml. At the same time, the weighed glass plates, held in small supporting clamps, were immersed in a substantially vertical position in the α-amylase/detergent solution, which was then stirred for 15 minutes at 55°C. The glass plates were then removed from the α-amylase/detergent solution, rinsed with distilled water, dried at 60°C in a drying cabinet and re-weighed. The performance of the α-amylase in question [expressed as an index relative to a chosen reference α-amylase (index 100) - in the present case the parent α-amylase having the amino acid sequence shown in SEQ ID No. 1] was then determined from the difference in weight of the glass plates before and after treatment, as follows:

$$\text{Index} = \frac{\text{weight loss for plate treated with } \alpha\text{-amylase}}{\text{weight loss for plate treated with reference}} \cdot 100$$

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Mini dishwashing test of deletion variant T183* + G184*

The above-described mini dishwashing test was performed at pH 10.5 with the parent α-amylase having the amino acid sequence shown in SEQ ID No. 1 and the thermally more stable deletion variant T183* + G184*. The test gave the following results:

parent α-amylase	Index: 100
T183* + G184*	Index: 120

The results clearly illustrate the beneficial effect on dishwashing performance which may be achieved using a variant according to the invention having improved thermal stability.

EXAMPLE 2

Construction of variants of the parent α-amylase having the amino acid sequence shown in SEQ ID No. 1

Description of plasmid pTVB106: The parent α-amylase having the amino acid sequence shown in SEQ ID No. 1 and variants thereof are expressed from a plasmid-borne gene, SF16, shown in Fig. 1. The plasmid, pTVB106, contains an origin of replication obtained from plasmid pUB110 (Gryczan et al., 1978) and the cat gene conferring resistance towards chloramphenicol. Secretion of the amylase is aided by the Termamyl™ signal sequence that is fused precisely, i.e. codon No.1 of the mature protein, to the gene encoding the parent α-amylase having the nucleotide and amino acid sequence (mature protein) shown in SEQ ID No. 4 and SEQ ID No. 1, respectively. The Termamyl promoter initiates transcription of the gene.

Plasmid pTVB106 is similar to pDN1528 (see laid-open Danish patent application No. 1155/94). Some unique restriction sites are indicated on the plasmid map in Fig. 1, i.e. SacII, BamHI, BstEII, AflIII, DraIII, XmaI, SalI and BglII.

Construction of variant M202T: The PCR overlap extension mutagenesis method is used to construct this variant (Higuchi et al., 1988). An approximately 350 bp DNA fragment of pTVB106 is amplified in a PCR reaction A using primers #7113 and mutagenic primer #6778. In a similar PCR reaction B, an approximately 300 bp DNA fragment is amplified using primers Y296 and #6779. The complete DNA fragment spanning the mutation site (M202) from primer #7113 to primer Y296 is amplified in PCR C using these primers and purified DNA fragments from reactions A and B.

PCR C DNA is digested with restriction endonucleases BstEII and AflIII, and the 480 bp fragment is ligated with plasmid pTVB106 digested with the same enzymes and transformed into a low-protease and low-amylase *Bacillus subtilis* strain (e.g. strain SHA273 mentioned in WO 92/11357).

Construction of variants T183* + G184* and R181* + G182*: The PCR overlap extension mutagenesis method is used to construct these variants (Higuchi et al., 1988). The mutagenic oligoneucleotides are synthesized using a mixture (equal parts) of C and G in one position; two different mutations can therefore be constructed by this procedure. An approximately 300 bp DNA fragment of pTVB106 is amplified in a PCR reaction A using primers #7113 and mutagenic primer #7449. In a similar PCR reaction B, an approximately 400 bp DNA fragment is amplified using primers Y296 and #3811. The complete DNA fragment spanning the mutation site (amino acids 181-184) from primer #7113 to primer Y296 is amplified in PCR C using these primers and purified DNA fragments from reactions A and B.

PCR C DNA is digested with restriction endonucleases BstEII and AflIII and the 480 bp fragment is ligated with plasmid pTVB106 digested with the same enzymes and transformed into a low-protease and low-amylase *B. subtilis* strain (e.g. strain SHA273 mentioned in WO 92/11357). Sequencing of plasmid DNA from these transformants identifies the two correct mutations: i.e. R181* + G182* and T183* + G184*.

Primers:

#7113:
5' GCT GCG GTG ACC TCT TTA AAA AAT AAC GGC 3'

Y296:
5' CC ACC GCT ATT AGA TGC ATT GTA C 3'

#6779
5' CTT ACG TAT GCA GAC GTC GAT ATG GAT CAC CC 3'

#6778
5' G ATC CAT ATC GAC GTC TGC ATA CGT AAG ATA GTC 3'

#3811
5' TT A(C/G)G GGC AAG GCC TGG GAC TGG 3'

#7449
5'C CCA GGC CTT GCC C(C/G)T AAA TTT ATA TAT TTT GTT TTG 3'

EXAMPLE 3

Determination of oxidation stability of variant M202T relative to the parent α-amylase having the amino sequence shown in SEQ ID No. 1

The enzyme variant to be tested (in this case M202T; see, e.g., Example 2) was purified to homogeneity in a manner analogous to that described above for the parent α-amylase having the amino acid sequence shown in SEQ ID No. 1. The buffer was changed to 50mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH), pH 9,0. Hydrogen peroxide was added to the enzyme

solution to give a final concentration of 200mM H_2O_2, and the solution was then incubated at 40°C in a water bath.

After 2, 4, 6, 10, 15 and 30 minutes of incubation the residual α-amylase activity was measured using the Phadebas assay described above. The residual activity in the samples was measured using 50mM Britton-Robinson buffer, pH 7,3, at 37°C (see Novo analytical publication AF207-1/1, available on request from Novo Nordisk A/S). The decline in activity was measured relative to a reference sample of the same enzyme at 0 minutes which was not incubated with hydrogen peroxide (100% activity).

The percentage of initial activity as a function of time is shown in the table below for the parent enzyme and for the variant in question.

Time in min.	SEQ ID No.1	variant M202T
0	100	100
4	57	52
6	42	67
10	31	52
15	14	37
30	2	24

The variant M202T clearly exhibits significantly improved stability towards oxidation relative to the parent α-amylase in question.

Determination of thermal stability of the deletion variant T183* + G184* relative to the parent α-amylase having the amino sequence shown in SEQ ID No. 1

The enzyme variant to be tested (in this case T183* + G184*; see, e.g., Example 2) was purified to homogeneity in a manner analogous to that described above for the parent α-amylase having the amino acid sequence shown in SEQ ID No. 1. The buffer was changed to 50mM Glycine buffer, 0,1mM $CaCl_2$, pH 10,5. The enzyme solution was then incubated in a water bath at 55°C.

After 5, 10, 15 and 30 minutes of incubation the residual activity was measured using the Phadebas assay described above. The residual activity in the samples was measured using 50mM Glycine buffer, pH 7,3, 37°C (see Novo analytical publication AF207-1/1, available on request from Novo Nordisk A/S). The decline in activity was measured relative to a reference sample of the same enzyme at 0 minutes which was not incubated (100% activity).

min.	SEQ ID No. 1	T183* + G184*
0	100%	100%
5	77%	89%
10	68%	89%
15	70%	85%
30	37%	86%

The variant T183* + G184* clearly exhibits significantly improved stability at 55°C relative to the parent α-amylase in question.

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SEQUENCE DESCRIPTION: SEQ ID No. 1

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
20 25 30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
115 120 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
165 170 175

Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr

245 250 255

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
305 310 315 320

His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
435 440 445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Val Trp Val Lys Gln
485

SEQUENCE DESCRIPTION: SEQ ID No. 2

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
20 25 30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
85 90 95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
115 120 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
165 170 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala

245 250 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
370 375 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
435 440 445

Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Ile Trp Val Lys Arg
485

SEQUENCE DESCRIPTION: SEQ ID No. 3

```
1   AAPFNGTMMQ YFEWYLPDDG TLWTKVANEA NNLSSLGITA LWLPPAYKGT
    SRSDVGYGVY DLYDLGEFNQ KGAVRTKYGT KAQYLQAIQA AHAAGMQVYA     100
    DVVFDHKGGA DGTEWVDAVE VNPSDRNQEI SGTYQIQAWT KFDFPGRGNT
    YSSFKWRWYH FDGVDWDESR KLSRIYKFRG IGKAWDWEVD TENGNYDYLM     200
    YADLDMDHPE VVTELKSWGK WYVNTTNIDG FRLDAVKHIK FSFFPDWLSD
    VRSQTGKPLF TVGEYWSYDI NKLHNYIMKT NGTMSLFDAP LHNKFYTASK     300
    SGGTFDMRTL MTNTLMKDQP TLAVTFVDNH DTEPGQALQS WVDPWFKPLA
    YAFILTRQEG YPCVFYGDYY GIPQYNIPSL KSKIDPLLIA RRDYAYGTQH     400
    DYLDHSDIIG WTREGVTEKP GSGLAALITD GPGGSKWMYV GKQHAGKVFY
    DLTGNRSDTV TINSDGWGEF KVNGGSVSVW VPRKTTVSTI AWSITTRPWT     500
    DEFVRWTEPR LVAWP                                          515
```

SEQUENCE DESCRIPTION: SEQ ID No. 4

CATCATAATG GAACAAATGG TACTATGATG CAATATTTCG AATGGTATTT GCCAAATGAC 60

GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA 120

GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC 180

TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA 240

ACACGCAACC AGCTACAGGC TGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300

GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360

GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420

ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480

CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540

AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600

CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660

GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT 720

ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780

ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840

AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA 900

TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960

CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020

GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080

CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCCG 1140

GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AAACTTTTGC CTATGGTACG 1200

CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260

CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320

TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380

ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440

GTTTGGGTGA AGCAA 1455

SEQUENCE DESCRIPTION: SEQ ID No. 5

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60

GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120

GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATGGAGCC 180

TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240

ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300

GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360

GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420

ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480

CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540

CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600

TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660

GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720

ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780

ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840

AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900

TCAAATAGTG GAGGCAACTA TGACATGGCA AAACTTCTTA ATGGAACGGT TGTTCAAAAG 960

CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA 1020

GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAAGAGAA 1080

CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140

GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200

CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260

CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320

TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380

ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440

ATTTGGGTGA AACGA 1455

SEQUENCE DESCRIPTION: SEQ ID No. 6

```
1   GCCGCACCGT TTAACGGCAC CATGATGCAG TATTTTGAAT GGTACTTGCC
    GGATGATGGC ACGTTATGGA CCAAAGTGGC CAATGAAGCC AACAACTTAT     100
    CCAGCCTTGG CATCACCGCT CTTTGGCTGC CGCCCGCTTA CAAAGGAACA
    AGCCGCAGCG ACGTAGGGTA CGGAGTATAC GACTTGTATG ACCTCGGCGA     200
    ATTCAATCAA AAAGGGACCG TCCGCACAAA ATACGGAACA AAAGCTCAAT
    ATCTTCAAGC CATTCAAGCC GCCCACGCCG CTGGAATGCA AGTGTACGCC     300
    GATGTCGTGT TCGACCATAA AGGCGGCGCT GACGGCACGG AATGGGTGGA
    CGCCGTCGAA GTCAATCCGT CCGACCGCAA CCAAGAAATC TCGGGCACCT     400
    ATCAAATCCA AGCATGGACG AAATTTGATT TTCCCGGGCG GGGCAACACC
    TACTCCAGCT TTAAGTGGCG CTGGTACCAT TTTGACGGCG TTGATTGGGA     500
    CGAAAGCCGA AAATTGAGCC GCATTTACAA ATTCCGCGGC ATCGGCAAAG
    CGTGGGATTG GGAAGTAGAC ACGGAAAACG GAAACTATGA CTACTTAATG     600
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    TGTCTTTGTT TGATGCCCCG TTACACAACA AATTTTATAC CGCTTCCAAA     900
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    AGATCAACCG ACATTGGCCG TCACCTTCGT TGATAATCAT GACACCGAAC    1000
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    TACGCCTTTA TTCTAACTCG GCAGGAAGGA TACCCGTGCG TCTTTTATGG    1100
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    TGAAAAACCA GGATCCGGAC TGGCCGCACT GATCACCGAT GGGCCGGGAG    1300
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    GACCTTACCG GCAACCGGAG TGACACCGTC ACCATCAACA GTGATGGATG    1400
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    GGTGAATTCG TCCGTTGGAC CGAACCACGG TTGGTGGCAT GGCCTTGA      1548
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CLAIMS

1. A variant of a parent α-amylase, which parent α-amylase (i) has one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively; or (ii) displays at least 80% homology with one or more of said amino acid sequences, and/or displays immunological cross-reactivity with an antibody raised against an α-amylase having one of said amino acid sequences, and/or is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding an α-amylase having one of said amino acid sequences; in which variant:

(a) at least one amino acid residue of said parent α-amylase has been deleted; and/or

(b) at least one amino acid residue of said parent α-amylase has been replaced by a different amino acid residue; and/or

(c) at least one amino acid residue has been inserted relative to said parent α-amylase;

said variant having α-amylase activity and exhibiting at least one of the following properties relative to said parent α-amylase: increased thermostability; increased stability towards oxidation; reduced Ca^{2+} dependency.

2. A variant according to claim 1, wherein at least one oxidizable amino acid residue of said parent α-amylase has been deleted or has been replaced by a different amino acid residue which is less susceptible to oxidation than said oxidizable amino acid residue.

3. A variant according to claim 2, wherein said oxidizable amino acid residue is selected from the group consisting of methionine, tryptophan, cysteine and tyrosine.

4. A variant according to claim 2 or 3, wherein said oxidizable amino acid residue is a methionine which is, or which is equivalent to, M9, M10, M105, M202,

M208, M261, M309, M382, M430 or M440 of the amino acid sequence shown in SEQ ID No. 1.

5. A variant according to claim 4, comprising a methionine substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: M9L; M10L; M202L,T,F,I,V; M261L; M309L; M382L; M430L; M440L.

6. A variant according to any one of claims 3-5, wherein a said methionine residue has been replaced by threonine.

7. A variant according to any one of the preceding claims, wherein at least one amino acid which is, or which is equivalent to, F180, R181, G182, T183, G184 or K185 of the amino acid sequence shown in SEQ ID No. 1 has been deleted.

8. A variant according to claim 7, wherein the deleted amino acids are, or are equivalent to, any two of said amino acid residues.

9. A variant according to claim 8, wherein the deletions are, or are equivalent to, R181* + G182*; or T183* + G184*.

10. A variant according to any one of the preceding claims, comprising an amino acid substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: P260E; R124P; M105F,I,L,V; M208F,W,Y; L217I; V206I,L,F.

11. A variant according to any one of the preceding claims, comprising an amino acid substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: Y243F; K108R; K179R; K239R; K242R; K269R; D163N; D188N; D192N; D199N; D205N; D207N; D209N; E190Q; E194Q; N106D.

12. A DNA construct comprising a DNA sequence encoding an α-amylase variant according to any one of claims 1-11.

13. A recombinant expression vector which carries a DNA construct according to claim 12.

14. A cell which is transformed with a DNA construct according to claim 12 or a vector according to claim 13.

15. A cell according to claim 14, which is a microorganism.

16. A cell according to claim 15, which is a bacterium or a fungus.

17. A cell according to claim 16, which is a grampositive bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a gramnegative bacterium such as *E.coli*.

18. A method of producing an α-amylase variant according to any one of claims 1-11, wherein a cell according to any one of claims 14-17 is cultured under conditions conducive to the production of the α-amylase variant, and the α-amylase variant is subsequently recovered from the culture.

19. Use of an α-amylase variant according to any one of claims 1-11 for washing and/or dishwashing.

20. A detergent additive comprising an α-amylase variant according to any one of claims 1-11, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

21. A detergent additive according to claim 20, comprising 0.02-200 mg of enzyme protein per gram of the additive.

22. A detergent additive according to claim 20 or 21, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

23. A detergent composition comprising an α-amylase variant according to any one of claims 1-11.

24. A detergent composition according to claim 23, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

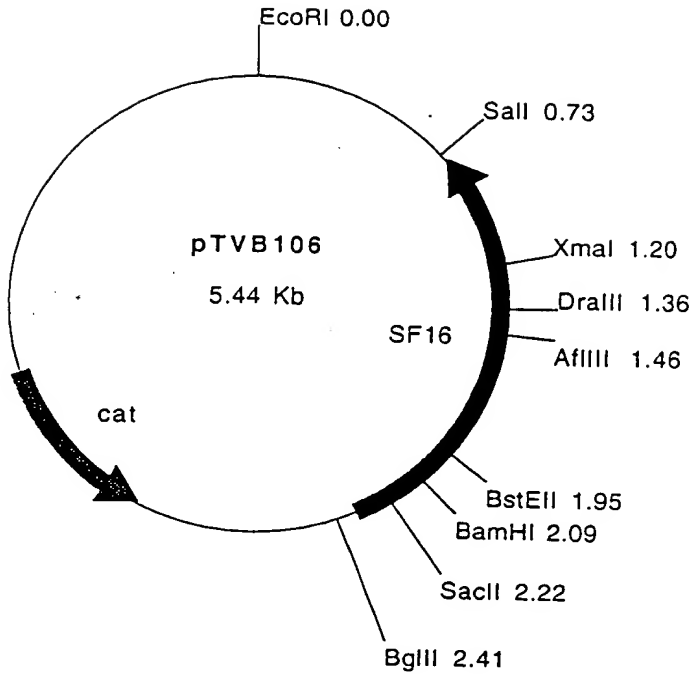
25. A manual or automatic dishwashing detergent composition comprising an α-amylase variant according to any one of claims 1-11.

26. A dishwashing detergent composition according to claim 25, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

27. A manual or automatic laundry washing composition comprising an α-amylase variant according to any one of claims 1-11.

28. A laundry washing composition according to claim 27, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

29. Use of an α-amylase variant according to any one of claims 1-11 for textile desizing.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SalI 0.73
pTVB106
5.44 Kb
XmaI 1.20
DraIII 1.36
SF16
AflIII 1.46
cat
BstEII 1.95
BamHI 2.09
SacII 2.22
BglII 2.41

Fig. 1